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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Numerous studies have identified a variety of morphological changes in the cell nuclei that can be correlated with cancer. We have been interested in the function of a specific nuclear compartment, interchromatin granule clusters (IGCs), and in determining if changes in its protein composition occur in breast cancer cells. We have previously identified 138 proteins from mouse IGCs which includes proteins involved in pre-mRNA processing. Using this as basis, we went on to establish an approach to purify the IGCs from suspension tissue culture cells (HeLa) and DU4475 (HTB-123), a breast cancer suspension cell line. Using several antibodies to pre-mRNA splicing factors (SF2/ASF, 3C5) we found a clear enrichment of these proteins in DU4475 IGCs. We also analyzed the DU4475 IGCs by 2-D gel electrophoresis. We observed some differences in the protein composition of IGCs between HeLa and breast cancer cells. Work is underway to characterize these differences. In addition, we have identified a novel potential non-coding RNA from sub-nuclear compartment from mouse liver IGC enriched in mammary tumor cells. Work is in progress for further characterization of this RNA.

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Table of Contents

Cover..... 1

SF 298..... 2

Introduction..... 4

Body..... 4

Key Research Accomplishments..... 8

Reportable Outcomes..... 8

Conclusions..... 8

References..... 9

Appendices..... 9

Introduction

Cancer is diagnosed by examining architectural alterations to cells and tissues. Alterations in nuclear structure are among the most universal of these and include variations in nuclear shape and changes in the internal organization of the nucleus including the morphology of sub-nuclear compartments. Generally cell nuclei contain numerous distinct sub-structures including nucleoli, Cajal bodies, gems, PML bodies and interchromatin granule clusters (IGCs), in addition to chromatin, some of which have been shown to be altered in number and/or appearance in tumors cells (Spector, 1993; Dyck et al., 1994; Ochs et al., 1994; Lamond and Earnshaw, 1998; Spector, 2001). IGCs or nuclear speckles are an important nuclear structure that plays a pivotal role in coupling RNA polymerase II transcription and pre-mRNA splicing, by modifying/supplying splicing factors to active sites of transcription (Sacco-Bubulya and Spector, 2002). Previously we have biochemically purified IGCs from mouse liver nuclei (Mintz et al., 1999). The mass spectrometry analysis identified ~138 proteins most of which are involved in various aspects of pre-mRNA processing. We also identified several proteins that have been previously shown to be involved in maintaining the structural integrity of the cell. Most interestingly, we also found 16 new proteins in our analysis for which no biological information is available, except for sequence information.

BODY

One of the major emphases of this present work was to purify the IGCs from cancerous and non-cancerous breast cells and to compare the candidate proteins that are modulated, either up-regulated or down-regulated in these cells. In the previous year we were successful in purifying the IGCs from a suspension HeLa cell line.

IGCs in breast cancer cell lines:

Prior to purifying the IGCs from breast cells I confirmed the presence of these sub-nuclear structures in breast cancer cells. I carried out immunostaining in MCF-7 cells using various markers of speckles, including B", an integral component of the U2 snRNP complex, SF2/ASF and SC35, well characterized non-snRNP splicing factors belonging to SR family. The comparison of immunostaining using the above antibodies in HeLa (Fig. 1) and MCF7 (Fig. 2) revealed that B" gave a characteristic nuclear speckled appearance in both the cell-lines (arrowheads in Fig. 1 and 2). In addition, in both cell lines B" also stained another prominent nuclear structure, the Cajal bodies (arrows in Fig. 1 and 2). Similarly, SF2/ASF and SC35 also showed a comparable pattern between the two cell types (Compare Fig. 1 & Fig. 2).

Biochemical purification of IGCs from breast cancer cell line:

As had been previously mentioned in the last annual report, it would be an enormous task to grow cell-lines as a monolayer (MCF-7 and HBL-100) at a density of 6 X 10^9, so as to be able to purify IGCs. Thus, as an alternative we proposed to grow another human breast cancer cell line DU4475 (ATCC number: HTB-123) as a suspension culture, which would allow us to achieve the cell density necessary for sufficient IGC purification. DU4475 is an epithelial cell-line that does not grow as a monolayer, but grows as aggregated clusters, often referred to as "serpentine". We were not successful in adapting these cells to grow as suspension culture in round-bottom flasks, since the stirring of the cells resulted in breakage of the clustered cells causing increased cell-death. Since this method failed even after several endeavors, we tried culturing these cells in T-flasks. After several passages, the cells adapted to the T-flask method and we were successful in achieving the cell-density required for IGC purification.

The procedure to purify the IGCs from these cells was similar to what has been standardized for HeLa IGCs (see Fig. 3). Immunoblotting of various fractions

purified from DU4475 cells using antibody 3C5, which specifically recognizes phosphorylated epitopes of the SR proteins (family of proteins that are enriched in IGCs) showed a clear enrichment in the IGC fraction compared to other fractions (Fig. 5), a similar result to what was observed for HeLa cells (Fig. 4). Surprisingly one of the SR proteins, SRp20 was not enriched in the IGCs of DU4475, unlike the HeLa cells (Compare Fig.4, and Fig. 5, lanes IGC). We belive this is not a result of low expression of SRp20 in DU4475, rather it seems to be more sensitive to salt extraction in this cell-line and accumulates in the salt extracted supernatants (See lanes DS1 and DS2, in Fig.5; and compare with DS1 and DS2 of HeLa in Fig. 4). SF2/ASF showed enrichment in IGCs similar to what has been observed in HeLa cells. The hnRNP proteins and lamins, proteins known not to enriched in IGCs, were low in the IGC fraction as compared to the nuclei, suggesting that the IGC purification showed enrichment of only the bonafide speckle proteins.

Thus far, we have not been successful in growing a non-cancerous breast cells in suspension, so as to purify the IGCs from them. In the present situation, we decided to run the IGC fraction from DU4475 and HeLa on a 2-dimensional SDS PAGE for a direct comparison. The silver staining of the 2-D gel showed that the two cell-lines had a highly similar profile (Fig. 6, see the white circles for comparison). However, we observed a few spots which were different in the two cell-lines, for example a ~80kDa protein which was enriched in HeLa and absent in the DU4475 cell line (Black circles). Also, a ~20kDa protein was enriched in DU4475, but seemed to be absent in HeLa cells. We are presently purifying more IGCs from both of these cell-lines and reconfirming the observations and subsequently will be sequencing the different spots by MALDI-TOF for identification of these proteins. It could however be possible, that these differences merely reflect a difference in the cell-lineages.

POLY A+ RNA ISOLATION FROM IGCs:

In the last few years, much data has accumulated showing that various non-translatable non-coding RNA transcripts are present in different cell types. They are lacking a protein coding capacity and exert their functions mainly or exclusively at the RNA level. Such RNAs are known to play versatile roles in various aspects of nuclear function such as, chromosome silencing, protein trafficking, as a structural framework to nuclear domains (Eddy, 1999; Prasanth et al, 2000) and are even connected with well known developmental, neurobiological disorders and cancer (Askew and Xu, 1999; Volker et al, 2001). Work from several groups has pointed to the presence of such a population of stable poly A+ RNA in IGCs where they are hypothesized to serve as a structural scaffold of the IGCs (Cheniclete and Bendayan 1990; Thiry, 1993; Huang et al, 1994). In addition to examining the protein components of IGC from breast cancer cells, we also envisage understanding the intriguing link between this class of non-coding RNAs, their role in maintaining the structural integrity of IGCs, and their characterization in carcinogenesis.

As an initial part of this study we had purified poly A+ RNA from mouse liver IGCs and cDNA was synthesized using oligo dT primers, and a pBluescript KS based cDNA library was constructed. We sequenced and characterized several clones, some of which showed novel localization pattern as discernable by RNA *in situ* hybridization studies in NIH3T3 cell lines and mouse embryonic fibroblasts. One of the clones (45.SL) of ~700 bp was interesting with respect to its sequence and localization pattern in the nucleus. The sequencing data suggests that the 700bp fragment did not show any homology to any known sequence and did not have any strong protein-coding frame. The *in situ* results showed that it stained several nuclear foci generally 20-40 per nucleus in various mouse cell-lines tested and these foci were distributed in a non-random fashion (Fig. 7a-b). Dual localization studies of this RNA and speckle protein SF2/ASF revealed that some of these RNA foci partially overlapped at the periphery of the speckles (Fig. 7c-e). Northern blot analysis on different cell-lines revealed that it encodes for a ~8kb transcript which is specific to mouse (Fig. 8). Interestingly,

other than liver tissue (fig. 8 lane-1) this RNA is highly enriched in mouse breast cancer cell-line, C127I, a mammary tumor cell line (fig. 8 lane 5), whereas it was expressed poorly in cell-lines from different tissue lineages.

KEY RESEARCH ACCOMPLISHMENTS

- Successful purification of IGCs from breast cancer cell-line, DU4475
- Immunoblot analysis to confirm IGC fractionation from the breast cancer cell line
- 2-D gel electrophoresis for comparison of HeLa and DU4475 IGCs
- Identification of a novel potential non-coding RNA from sub-nuclear compartment from mouse liver IGC enriched in mammary tumor cells.

REPORTABLE OUTCOMES

- A copy of the abstract presented in the "Era of Hope" Dept. of Defence Breast cancer research program meeting in Florida (September 25-28, 2002) is attached.

CONCLUSIONS

We have successfully purified IGCs from the breast cancer cell-line, DU4475. The quality of the IGC fraction from this has been confirmed by immunoblot analysis. Further, it was observed that the proteins, which were enriched in HeLa IGCs were found to be enriched in the DU4475 IGC fractions (splicing factors and RNA processing factors). 2-D gel analysis for comparison between HeLa and DU4475 revealed a few interesting differences. Work is underway to further characterize these results.

We have also identified a novel potential non-coding RNA from the sub-nuclear compartment from mouse liver IGCs. This RNA was expressed in high

abundance in a mouse mammary tumor cell line. Work is in progress to further characterize this RNA.

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APPENDICES

Figure 1: Immunostaining of HeLa cells using antibodies against various speckle proteins.

Figure 2: Immunostaining of MCF-7 cells using antibodies against various speckle proteins.

Figure 3: Diagrammatic representation of the procedure for biochemical purification of IGCs from cell lines.

Figure 4 & 5: Immunoblots illustrating the enrichment of splicing factors in the IGC fraction from HeLa (figure 4) and DU-4475 (figure 5) cells.

Figure 6: 2D gel analysis of IGC protein fractions from HeLa and DU-4475 cells.

Figure 7: RNA *in situ* hybridization analysis using nick translated 45.SL probes in NIH 3T3 cells (figure 7 a & b). Figure 7 c-e illustrates dual localization of RNA *in situ* using 45.SL probe and immunostaining using SF2/ASF antibody in NIH3T3 cells.

Figure 8: Cell line Northern analysis with 45.SL and β-actin probe

FIGURE LEGENDS:

Figure 1: Immunostaining of HeLa cells using antibodies against various speckle proteins B" (a-b), SF2/ASF (c-d) and SC-35 (e-f). Arrowhead depicts speckles and arrows (in b) denote Cajal bodies. Chromatin was stained using DAPI (a, c and e).

Figure 2: Immunostaining of MCF-7 cells using antibodies against various speckle proteins B" (a-b), SF2/ASF (c-d) and SC-35 (e-f). Arrowhead depicts speckles and arrows (in b) denote Cajal bodies. Chromatin was stained using DAPI (a, c and e).

Figure 3: Schematic representation of the biochemical purification of IGC fraction from cell-lines.

Figure 4: Immunoblot analysis from various biochemical fractions during HeLa IGC purification using antibodies 3C5, SF2/ASF, hnRNPA1 and Lamin B1.

Coomassie stained gel shows the level of protein loaded in each fraction. See Figure 3 for details of each fraction used.

Figure 5: Immunoblot analysis from various biochemical fractions during DU4475 IGC purification using antibodies 3C5, SF2/ASF, hnRNPA1 and Lamin A/C. See Figure 3 for details of each fraction used.

Figure 6: 2D gel analysis of IGC protein fractions from HeLa and DU-4475 cells. White circles denote similar spots. Black and red spots are the one that are HeLa and DU4475 specific spots respectively.

Figure 7: RNA *in situ* analysis using nick translated 45.SL probes in NIH 3T3 cells (figure 7 a & b). Figure 7c-e illustrates dual localization of RNA *in situ* hybridization using 45.SL probe and immunostaining using SF2/ASF in NIH3T3 cells. Chromatin was stained using DAPI.

Figure 8: Northern blot analysis using random labeled 45.SL probe in various cell lines from mouse (lanes 1-5), rat (6), hamster (7-8) and human (9-14). β-actin was used as loading control.

ANALYSIS OF PROTEIN COMPONENTS OF INTERCHROMATIN GRANULE CLUSTERS FROM BREAST CANCER CELLS

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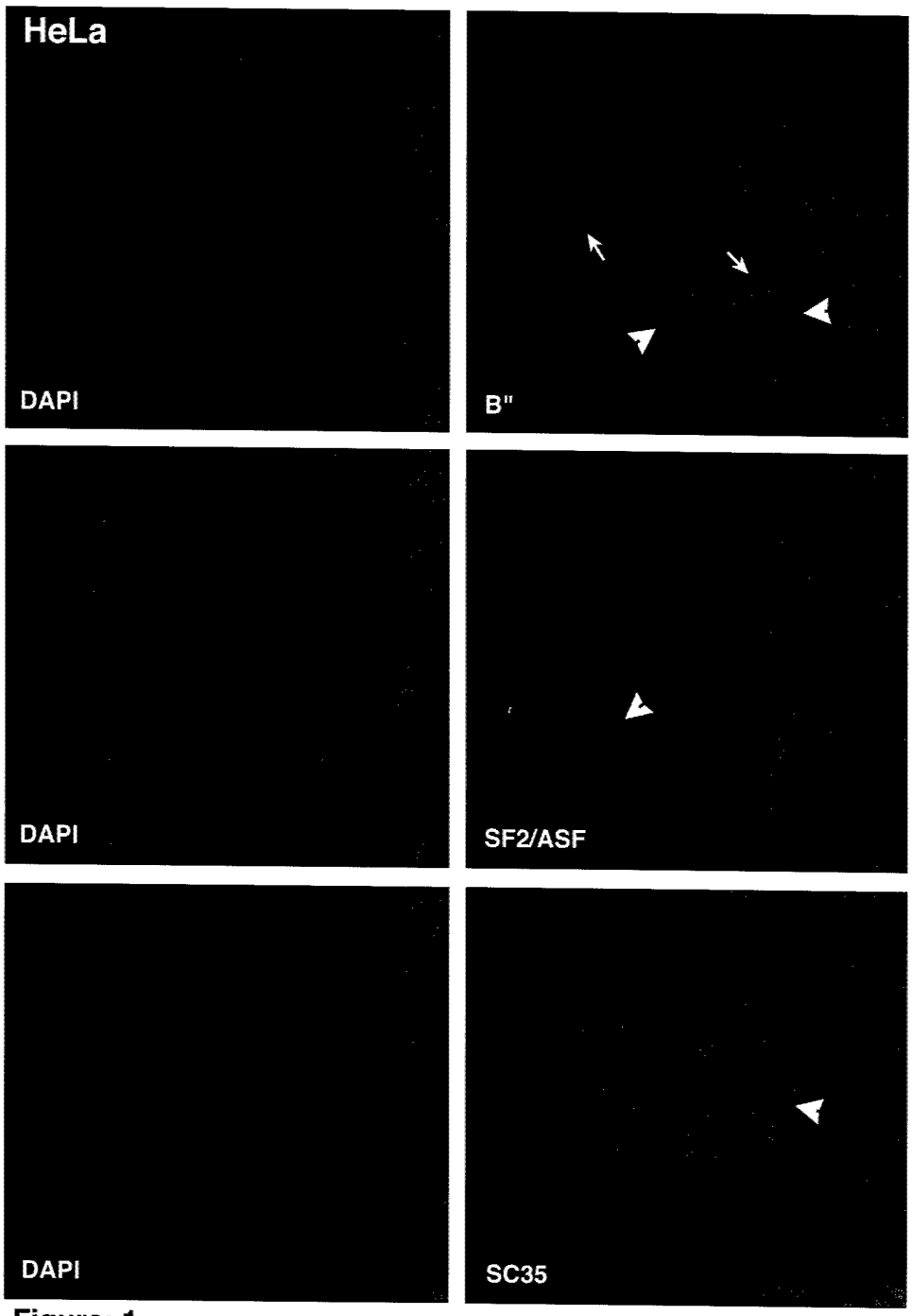
Numerous studies have identified a variety of morphological changes in the cell nuclei that can be correlated with cancer. We have been interested in the function of a specific nuclear compartment, interchromatin granule clusters (IGCs), and in determining if changes in its protein composition occur in breast cancer cells. Thus far, a large number of proteins involved in gene expression including RNA polymerase II, pre-mRNA splicing factors, as well as several kinases and several cancer related proteins have been reported to be localized to the IGCs. In the present study we set out to identify the proteome of normal IGCs and then to determine if differences occur in those purified from breast cancer cells. Since these structures are enriched in the members of the gene expression machinery we hope that our screen will identify proteins that may have a role in the breast cancer process. In order to develop this approach we first set out to identify the entire protein composition of the IGC fraction isolated from mouse liver nuclei as we could obtain large amounts (1mg) of a fraction enriched in this structure. Our purified fraction was analyzed using liquid chromatography and tandem mass spectrometry (LC-MS/MS) combined with peptide sequence analysis and database searching. Using this approach, we have identified 138 proteins many of which were previously known to localize to IGCs or whose functions are associated with IGCs. This includes proteins involved in pre-mRNA processing such as 5' capping proteins, splicing factors and 3' processing factors. Furthermore, our analysis identified several proteins that were recently identified by others as having roles in pre-mRNA splicing or to be localized to IGCs. Using this as basis, we went on to establish an approach to purify the IGCs from suspension tissue culture cells (HeLa) and then we used this approach to purify the IGCs from DU4475 (HTB-123), a breast cancer cell line that can grow in suspension culture. Using several antibodies to pre-mRNA splicing factors (SF2/ASF, 3C5) we found a clear enrichment of these proteins in DU4475 IGCs. This fraction is currently being analyzed by 2-D gel electrophoresis. This study will characterize the differences in the protein composition of IGCs between normal and breast cancer cells and aims to identify critical proteins involved in breast cancer.



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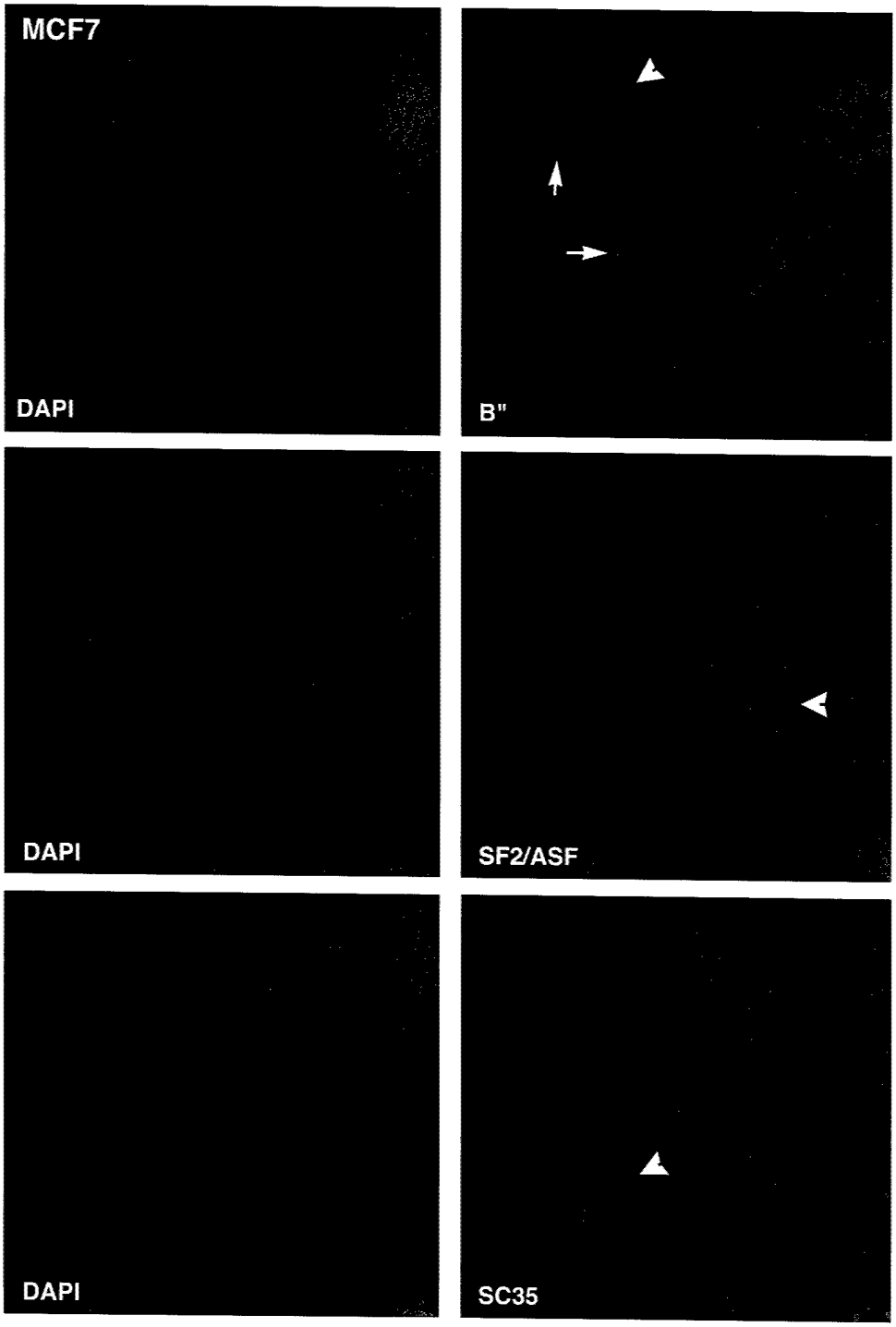

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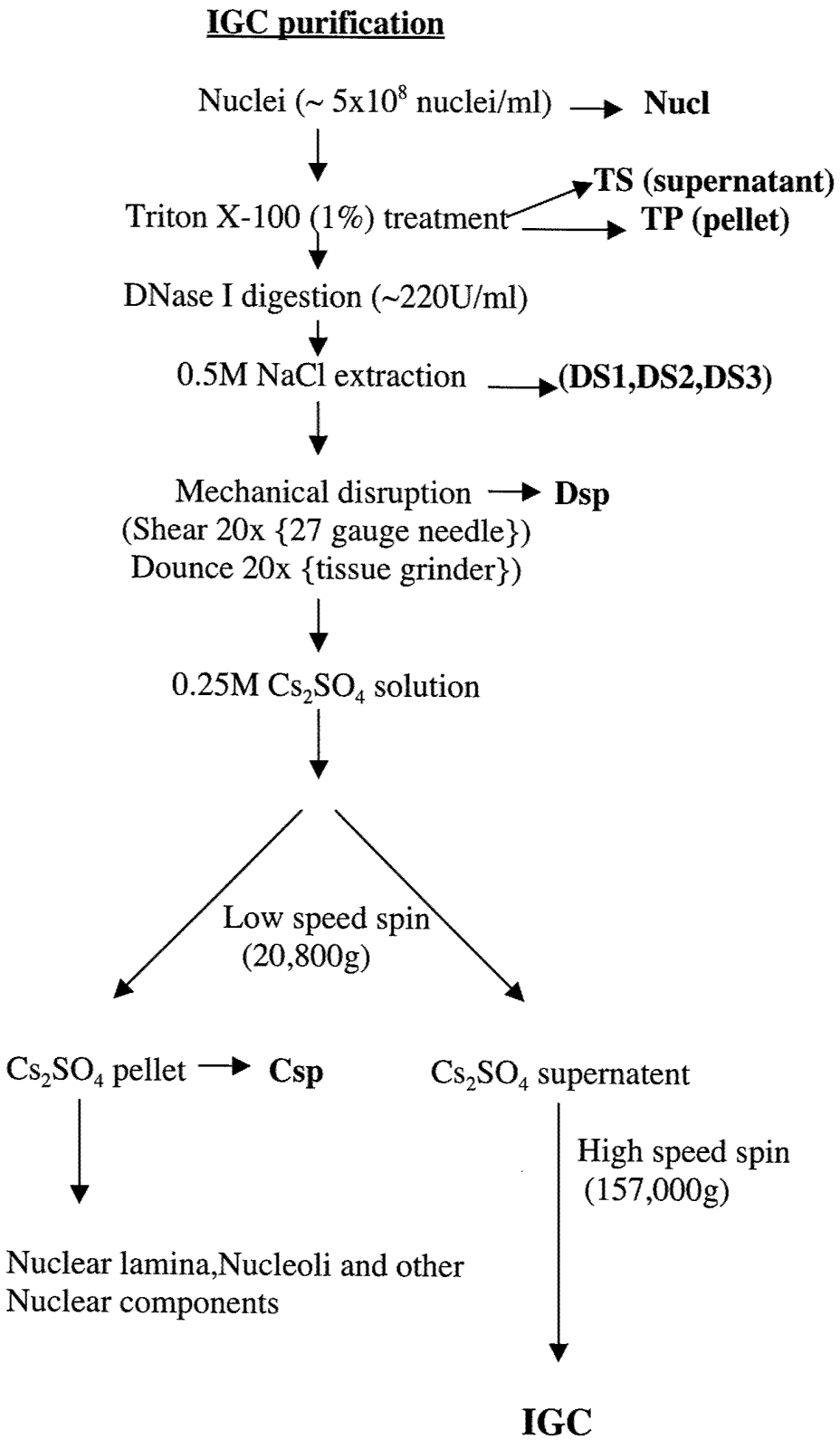
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TS (supernatant)
TP (pellet)
DNase I digestion (~220U/ml)
0.5M NaCl extraction
(DS1,DS2,DS3)
Mechanical disruption
Dsp
(Shear 20x {27 gauge needle})
Dounce 20x {tissue grinder})
0.25M Cs2SO4 solution
Low speed spin
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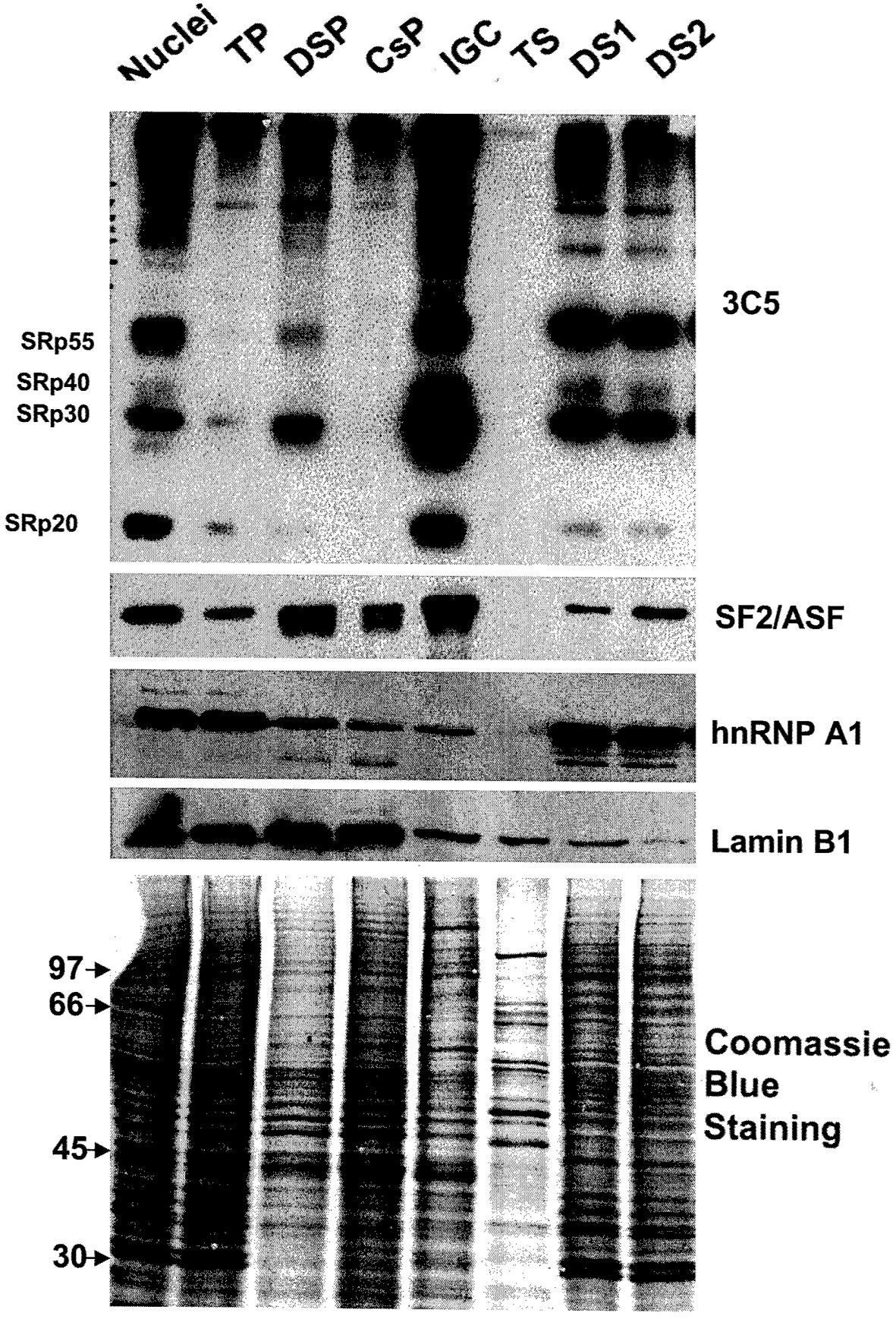
IGC

Figure: 3



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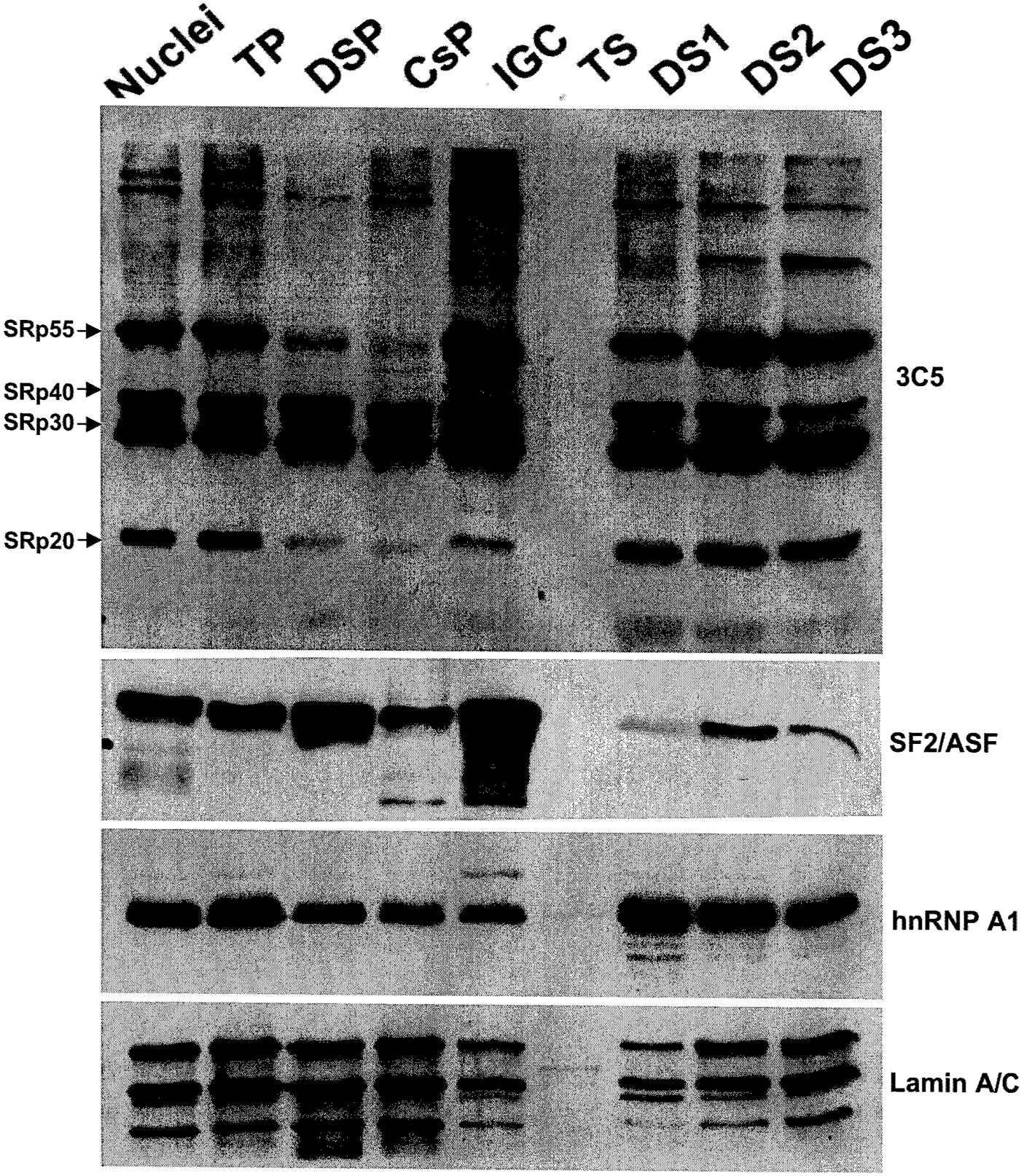

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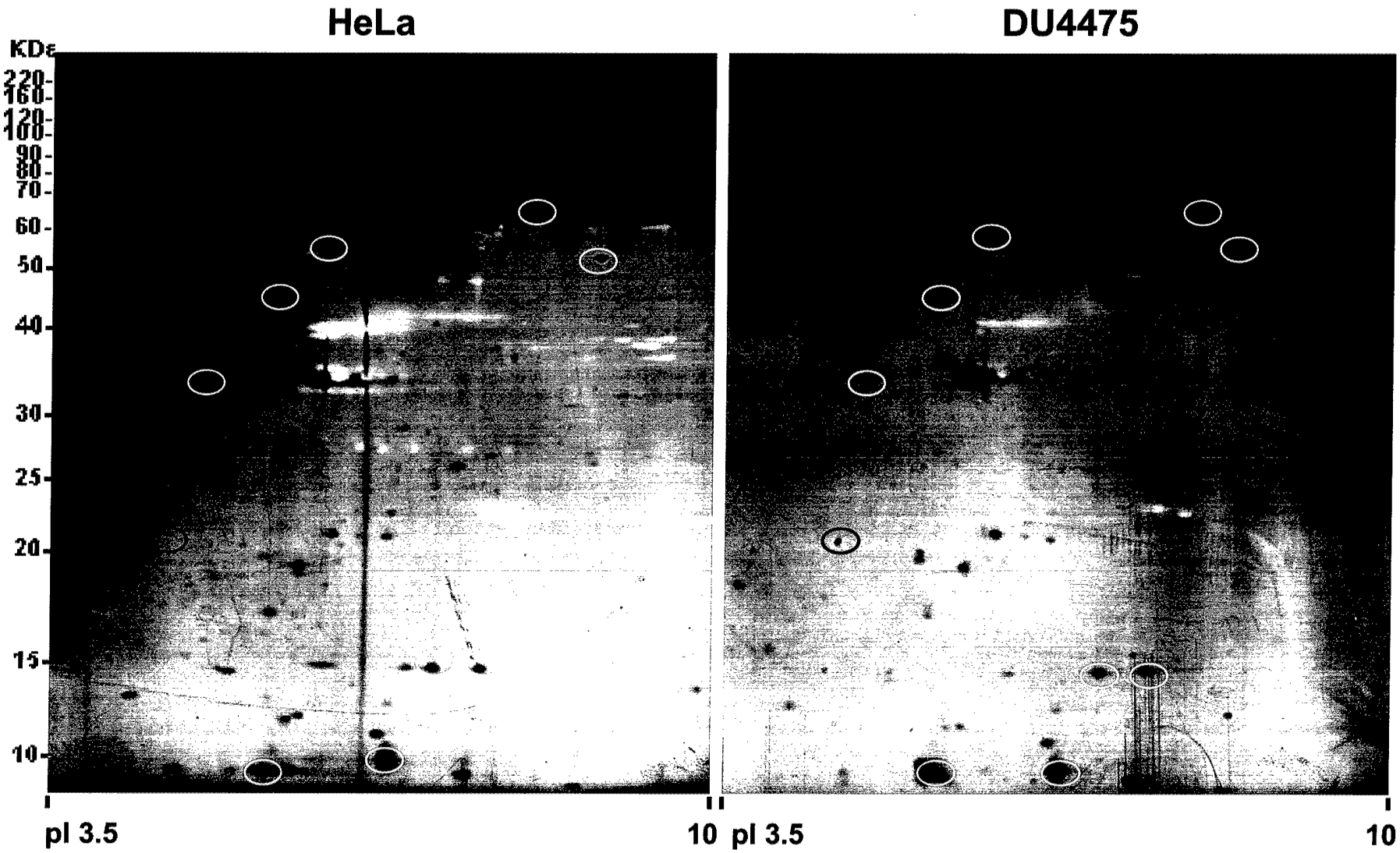
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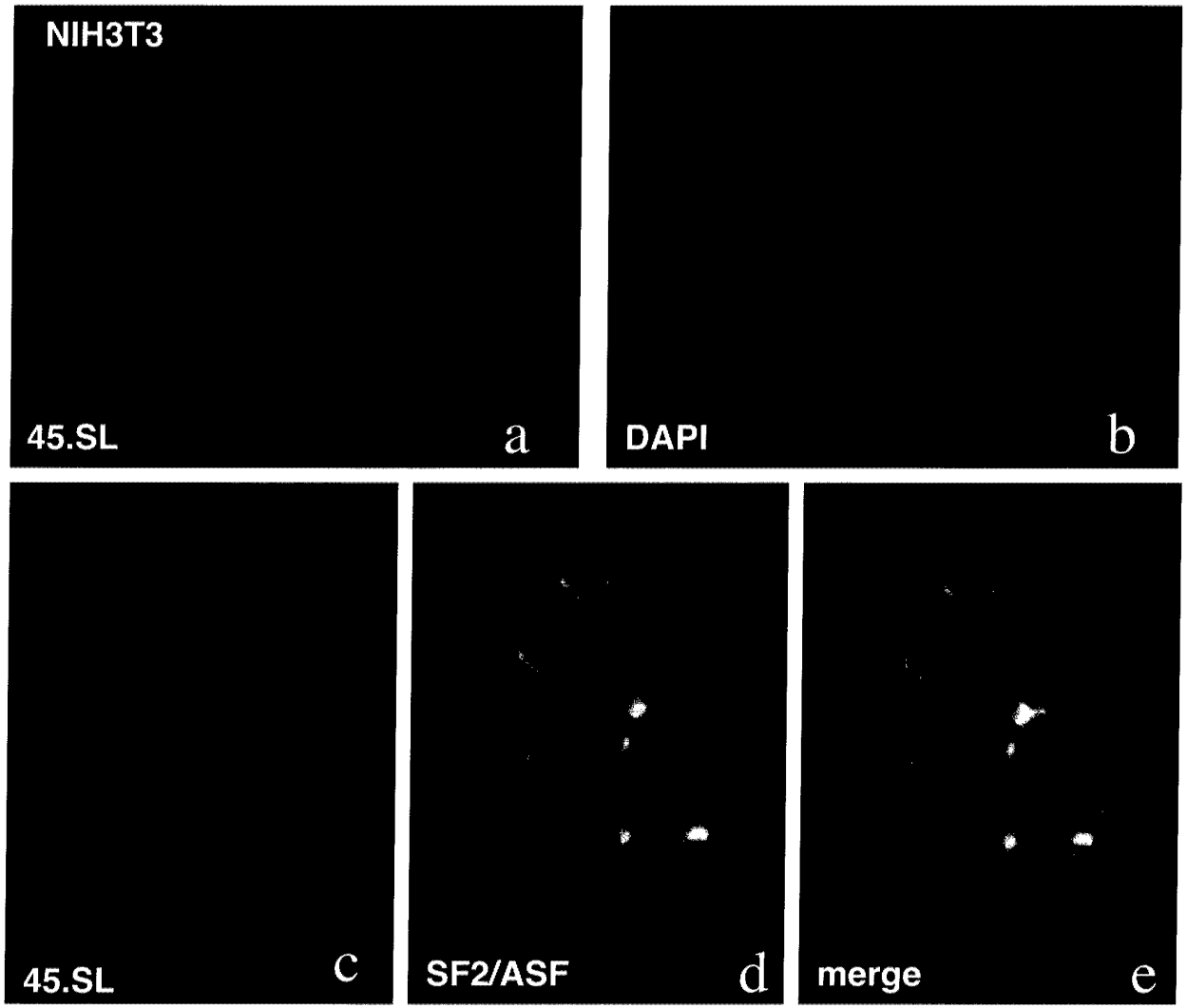
Figure: 6



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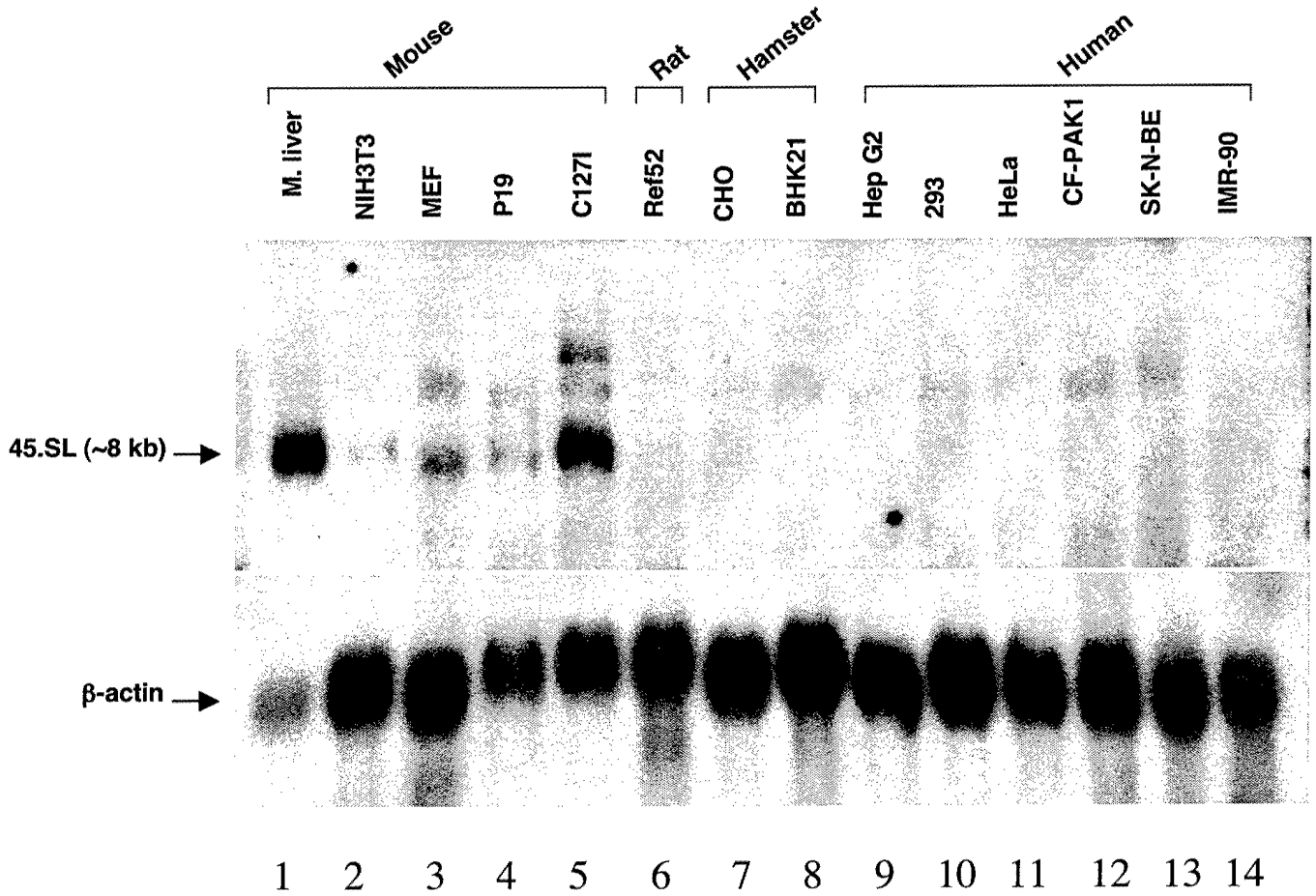
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β-actin
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Figure: 8